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RESEARCH

Contrasting biological responses of gingival fibroblasts and keratinocyte to blue and violet light irradiation: implications for photobiomodulation use in the therapeutic management of periodontal disease

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Abstract

Blue light is known to possess anti-microbial activity and has subsequently been proposed as a prophylactic treatment for the management of periodontal diseases. This study investigated the effect of blue light on cells from the soft gingival tissues within the oral cavity, with particular focus on its influence on the regulation of intracellular reactive oxygen species (ROS) production. Primary human gingival fibroblasts (pHGFs) and keratinocytes (pHGKs) were irradiated with either 457nm blue light or with 415nm violet light, with fluences 3–90 J/cm^2 and cell viability was assessed. The influence of blue light on ROS production was measured using 2',7'-dichlorodihydrofluorescein diacetate assay, in the presence and absence of ROS scavenger, N-acetylcysteine. Gene expression for a range of antioxidant genes was quantified and expression changes evaluated by western blot analysis. Following irradiation with blue light, pHGFs displayed minor reductions in cell viability across the fluence range, while pHGK proliferation and metabolic activity was enhanced following irradiation. Significant cytotoxic effects were seen in cells irradiated with violet light. Treatment of pHGFs with blue light induced significant ROS generation, which was inhibited by N-acetylcysteine. Only non-significant increases in antioxidant gene expression were identified for NQO1, GSR, GSS and KEAP1, in response to 36 and 60 J/cm2 doses. No corresponding changes in their corresponding protein levels were evident. Irradiation of gingival cells with 457 nm blue light produced negligible detrimental effects. Although ROS increased, this paper discusses how endogenous antioxidant defence mechanisms are sufficient to control any potential detrimental effects.

Keywords Blue light · Gingival tissues · Fibroblasts · Keratinocytes · Reactive oxygen species · Anti-oxidants · Cell viability · Periodontal disease

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Introduction

Black-pigmented bacteria and their metabolites present in oral biofilms, are regarded to be responsible for provoking host inflammatory responses that subsequently result in tissue destruction seen during periodontal diseases [1]. UV-free wavelengths bordering the violet and blue regions of the electromagnetic spectrum (400–500 nm) have been shown to exert anti-microbial effects on black-pigmented bacteria [2–4]. Indeed, a limited number of clinical trials have demonstrated potential for low levels of blue light to irradicate oral bacteria and the use of UV-free light as part of periodontal therapy [4–8]. Unlike light sources in the UV wavelengths, exposure to UV-free wavelengths are not

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considered to be associated with direct DNA damage and mutagenesis [9]. However, studies investigating the effects of blue light on cells in mucosal tissues lining the oral cavity, that would be exposed to any UV-light treatment, are limited.

Within bacteria, the damaging effects of UV-free blue light are mediated by endogenous porphyrin photoreceptors, stimulating the excessive and cytotoxic generation of reactive oxygen species (ROS) [3–5]. Photoreceptors, such as flavins, porphyrins, and nitrosated proteins in mammalian cells, function as key cofactors in mitochondrial redox electron transport chains [10–12]. Low-intensity blue light induces redox reactions, generating non-toxic ROS and nitric oxide that regulate signalling pathways involved in migration, proliferation, differentiation, inflammation, and apoptosis [13, 14]. However, when blue light levels are excessive, ROS and the associated oxidative stress can induce direct and indirect damage to cellular components, impairing cellular activity [15, 16].

Irradiation with broad-spectrum blue light (420–600 nm) have noted reductions in cell proliferation and migration in dermal and gingival fibroblasts, with no impact observed on cell viability [14, 17–19]. Similar effects are reported at lower fluences on gingival fibroblasts, suggesting wavelength, and not irradiance, was the primary determinant of response [19]. Supporting this hypothesis, Liebmann and co-workers [18] identified that blue light had no effects on keratinocytes and endothelial cells at 453 nm, while shorter wavelengths at 412–426 nm were cytotoxic at fluences of 33 J/cm^2. Collectively, the above studies point towards different tolerance levels between fibroblasts and keratinocytes.

Despite oral mucosal and dermal wounds proceeding through similar stages of healing, oral wounds are characterized by reduced inflammation, rapid healing and minimal scar formation; significantly contrasting to wound healing response for dermal wounds [20–26]. Gingival fibroblasts present a distinct "young" subpopulation within the oral mucosa which, when compared with dermal fibroblasts, differ in gene expression [24], intracellular wound healing response [25, 26], and appear to be more resistant to oxidative stress-related damage [22, 23]. These differences could suggest that oral and dermal fibroblasts respond differently to UV-free blue light irradiation. Therefore, the aim of this study was to investigate the cellular responses of primary human gingival fibroblasts and keratinocytes following irradiation with 457 nm blue light, with effects compared to shorter wavelengths of 418 nm in the violet spectrum. Significantly, recognising that UV-free light can stimulate ROS generation, this study considers the role for ROS and antioxidant gene expression in modulating the observed effects.

Materials and methods

LED light source and calibration

For the delivery of blue or violet light treatments, cells were exposed to blue (457 nm) or violet (418 nm) using a custom Light Emitting Diode (LED) array inserted into the roof of a black anodised aluminium chamber, ensuring consistent alignment and distance of cells from the light source (Philips Electronics Nederland B.V., Eindhoven, The Netherlands). Irradiance at the culture interface was calibrated using a Coherent PowerMax USB PS10 Measurement System, paired with PowerMax PC software V2.1.0. A 240 V power supply was provided to the LED array and amperage progressively increased until the irradiance recorded by the sensor reached 100 mW/cm or 300 mW/cm . Cells were irradiated for timed periods between 30 and 600 s, to provide fluences ranging from 3 to 90 J/cm (Fluence (J/cm) = Irradiance (mW/cm) x Time (s).

Gingival cell culture and irradiation

Primary human gingival fibroblasts (pHGFs) and primary human gingival keratinocytes (pHGKs) were sourced from ATCC/LGC Standards (Manassas, Virginia, USA). pHGF cells (healthy 60-year-old Caucasian female donor) were maintained in Dulbecco's Modified Eagle Medium (DMEM), 10% foetal calf serum (FCS, ThermoFisher Scientific, Paisley, UK), 100 units/mL penicillin G sodium, 0.1 µg/mL streptomycin sulphate and 0.25 µg/mL amphotericin (ThermoFisher Scientific). pHGKs (healthy 65-year-old Caucasian male donor) and were maintained in Dermal Cell Basal Medium (DCBM, ATCC), supplemented with a Keratinocyte Growth Kit (ATCC). All cells were seeded at 5,000 cells/cm^2 into clear multi-well culture plates. Serum free medium (SFM) replaced growth medium 24 h, prior to irradiation and was refreshed immediately prior to light treatments.

Cell viability assays

At 24 h post-blue treatment, AlamarBlue™ assay was utilised to assess the influence of blue or violet light upon gingival cell metabolic activity (ThermoFisher Scientific). LDH release (as a marker of cell necrosis) was measured using a CyQuant LDH Cytotoxicity Assay Kit (ThermoFisher Scientific), 24 h post-blue or violet light treatment, according to the manufacturer's protocol.

Generation of reactive oxygen species (ROS)

ROS generation was measured using a 2',7'-dichlrorodihydrofluorescein diacetate (H_2DCFDA) assay [27]. Following treatment with blue or violet light, cells were washed with sterile phosphate buffered saline (PBS), incubated with 10 µM H_2DCFDA (Sigma, Poole, Dorset, UK) for 30 min (37 °C/5% CO_2), then washed twice with PBS and fluorescence was recorded using a FLUOstar® Omega Plate Reader (BMG Labtech, Aylesbury, UK), at 494 nm excitation and 522 nm emission. Providing a negative control, N-acetylcysteine (NAC), a non-specific ROS scavenger, was added to the SFM 30 min prior to irradiation. Reduction in ROS was examined using the above H_2DCFDA protocol after light treatment.

Quantitative reverse transcription – polymerase chain reaction (RT-qPCR)

At 6 h and 24 h post-treatment with blue or violet light, total RNA was extracted from pHGF and pHGK cultures using a RNeasy Mini Kit (Qiagen, Manchester, UK). Complementary DNA (cDNA) libraries were synthesised using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, ThermoFisher Scientific). For quantification of antioxidant gene expression, master mixes comprised 10 µL Applied Biosystems™ TAQMAN® Fast Universal PCR Master Mix (2X), no AmpErase™ UNG, 4 µL RNase-free water, 1 µL Target gene primer or 1 µL endogenous house-keeping gene, *18s rRNA* (see Table 1 for detail of TAQMAN® genes). *18s rRNA* endogenous control TAMRA probes feature a 5' fluorescent reporter dye (VIC) and a 3' fluorescent quencher (TAMRA). All other gene targets used TaqMan MGB (minor groove binding) probes, featuring a 5' fluorescent reporter dye (FAM) and 3' nonfluorescent quencher (NFQ). cDNA amplification was performed using ViiA™ 7 Real-Time PCR System (Applied Biosystems™, ThermoFisher Scientific) with reaction conditions: 1 cycle of 95 °C for 20 s, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. Relative expression of target genes were determined according to the ΔΔCT method, as described by [28].

Table 1 TAQMAN® target primer probes and endogenous control primer used for analysis of antioxidant gene expression

Antioxidant protein	Gene Name	TAQMAN® Gene Expression Assay id
Nuclear Factor Erythroid 2-related Factor 2	*NFE2L2*	Hs00975961_g1
Kelch-like ECH-associated Protein 1	*KEAP1*	Hs00202227_m1
Glutathione Disulfide Reductase	*GSR*	Hs00167317_m1
Glutathione Synthetase	*GSS*	Hs00609286_m1
Glutathione Peroxidase 1	*GPX1*	Hs00829989_Gh
Glutathione Peroxidase 4	*GPX4*	Hs00989766_m1
Superoxide Dismutase 1	*SOD1*	Hs00533490_m1
Superoxide Dismutase 2	*SOD2*	Hs00167309_m1
NADPH Quinone Reductase	*NQO1*	Hs01045993_g1
Catalase	*CAT*	Hs00156308_m1
18 S ribosomal RNA	*18 S rRNA*	4310893E

Western blot analysis

At 6 h and 24 h post-treatment, cells were washed twice with ice-cold PBS and cell lysate was obtained by treating for 2 min with Pierce® radioimmunoprecipitation assay buffer (RIPA, ThermoFisher Scientific), supplemented with cOmplete™ Mini Protease Inhibitor Cocktail Mix (Sigma, UK). Resultant protein lysates were separated by SDS-PAGE gel electrophoresis on 4–15% mini-PROTEAN®TGX™ precast acrylamide gels and protein transferred to nitrocellulose membranes (GE Healthcare, Chalfont St Giles, Hertfordshire, UK), using the BioRad Mini Trans-Blot System (all BioRad, Watford, Hertfordshire, UK). Membranes were blocked by exposure for 1 h with 5% whole milk powder in TBST (20 mM Tris-HCl, 150 mM Sodium Chloride, 0.1% Tween 20, pH 7.6), then incubated for 1 h with the primary antibody, followed by TBST washing and 45 min incubation with the secondary antibody (all sourced from Abcam UK, Cambridge, UK); all antibodies diluted in blocking buffer; see Table 2 for details). Proteins were visualised using the Enhanced Chemiluminescent substrate (ECL, ThermoFisher Scientific) and the density of respective bands quantified using an iBright Imaging System with integrated iBright Analysis Software Version 4.0 (ThermoFisher Scientific, UK).

Statistical analysis

All experiments were performed in triplicate, with three internal repeats per experimental repeat and means with standard deviation (SD) were calculated. Statistical analyses were performed with GraphPad Prism9 Software 2022 (Version 10.4.2). Following tests for normality, statistical

Table 2 Details for primary and secondary antibodies used for the investigation of antioxidant protein levels by Western blot analysis

Target antigen	Antibody type	Abcam Catalogue No.	Dilution
GSR	Rabbit monoclonal 1° Ab	ab124995	1:500
KEAP1	Rabbit monoclonal 1° Ab	ab227828	1:500
NQO1	Rabbit monoclonal 1° Ab	ab80588	1:500
β-actin	Rabbit polyclonal 1° Ab	ab8227	1:20,000
HRP Preabsorbed	Goat polyclonal anti-rabbit 2° Ab	Ab7090	1:10,000

analysis of AlamarBlue™ and LDH results was performed using a Student t-test. Statistical significance in qPCR and H_2DCFDA results was determined via Two-Way ANOVA with Dunnett's multiple comparisons post-hoc test. Statistical analyses of qPCR data were performed upon ΔΔCT values. In all analyses, $p < 0.05$ was considered significant.

Results

Gingival fibroblasts and keratinocytes display differential cellular responses to blue (457 nm) treatment

Different metabolic responses were observed between pHGFs and pHGKs, when assessed 24 h post-irradiation with 457 nm blue light treatment (Fig. 1A and B, respectively). A significant reduction in metabolic activity was seen in pHGFs irradiated with 30 J/cm^2 (Fig. 1A, $p < 0.01$). Conversely, significant increases in metabolic activity were observed in pHGKs irradiated with 9, 36, 60 and 90 J/cm^2 (Fig. 1B, $p < 0.05$, $p < 0.01$). Dose dependency of response in both pHGFs and pHGKs was also evident, with higher doses resulting in the greater changes in metabolic activity. LDH release, used as a marker of cytotoxicity, was found to be significantly upregulated in pHGFs treated with 3, 9, 12, 36, 60 and 90 J/cm^2 of 457 nm blue light (Fig. 1C, $p < 0.01$). However, no significant changes in LDH levels were seen in pHGKs in response to blue light irradiation (Fig. 1D, all $p > 0.05$).

Influence of violet wavelengths (418 nm) on cellular proliferation and viability

Noting the effects of blue light (457 nm) treatment was more pronounced for pHGFs, the above results were further compared using similar metabolic and cytotoxic assays performed on gingival fibroblasts, following treatment with violet light (418 nm). Overall, irradiation of pHGFs with 418 nm resulted in a decrease in metabolic activity at higher

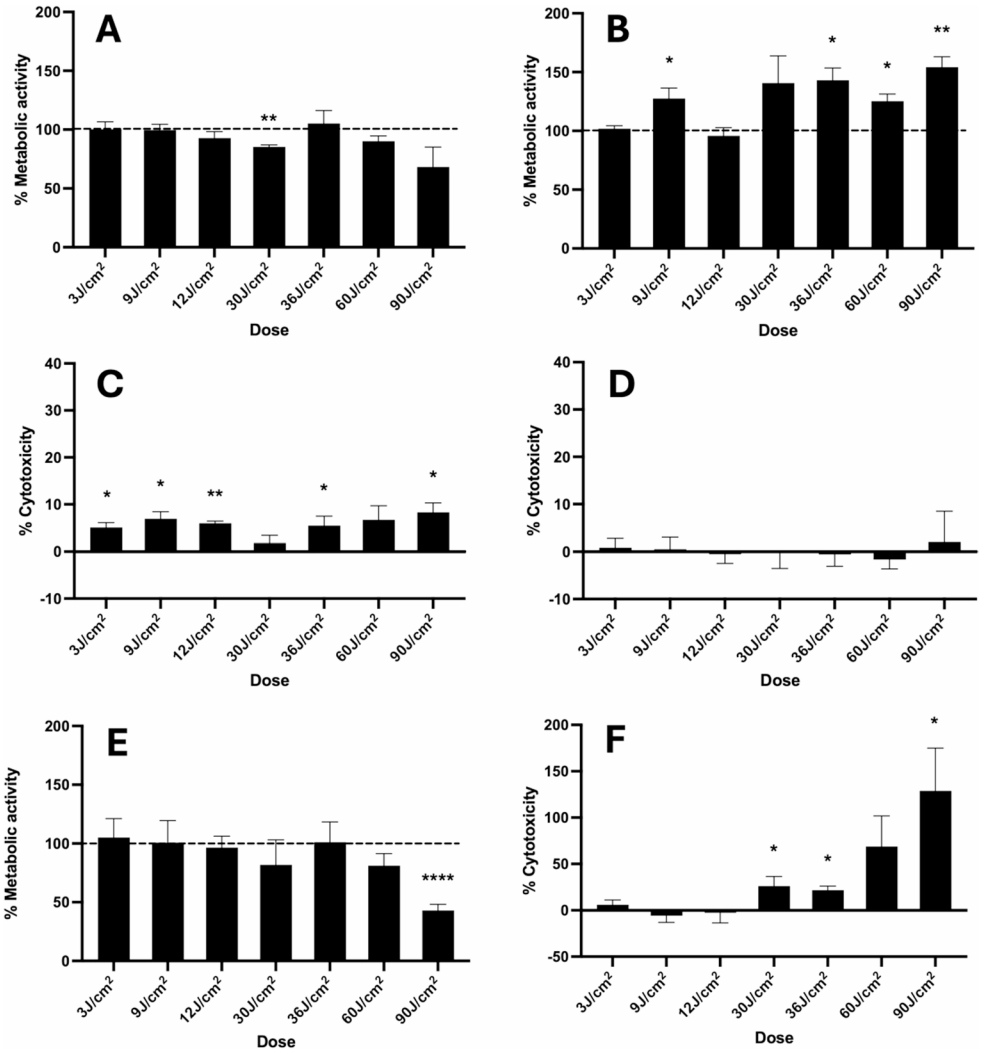

Fig. 1 Influence of blue (457 nm, A-D) and violet (418 nm, E-F) light upon the metabolic activity and cytotoxicity of pHGFs (A, C and E, respectively) and pHGKs (B, D and F, respectively). Cells were irradiated with different fluences of blue and violet light. At 24 h post-treatment, the metabolic activity of the cells was assessed using AlamarBlue™. Metabolic activity was expressed as a percentage change compared to untreated controls; where the untreated controls were normalised to 100%, represented by the dashed line. LDH release, a marker of cytotoxicity, was also assessed 24 h post-treatment and cytotoxicity calculated as the percentage change in LDH levels compared to untreated controls, normalised to 0%. N = 3, mean ± SD (*p < 0.05, **p < 0.01, ****p < 0.0001)

fluences, reaching significance at the highest dose (90 J/cm^2, Fig. 1E, $p < 0.0001$). A dose-dependent increase in cytotoxicity was seen in pHGFs treated with 418 nm, with significant increases seen in response to 30, 36 and 90 J/cm^2 (Fig. 1F, $p < 0.05$). Compared to cells irradiated with blue light (457 nm), violet light treatment resulted in a more detrimental responses on cell health.

Blue light treatment induces significant ROS generation

Significant increases in ROS levels were observed following the pHGFs treatment with blue light at 3, 36 and 60 J/cm^2 doses (Fig. 2A, $p < 0.001$). Data obtained for pHGKs irradiated with blue light also indicated a trend for elevated ROS levels with increasing treatment dose, although these changes were not statistically significant (Fig. 2B, $p > 0.05$). Addition of ROS scavenger, NAC, confirmed that ROS levels generated during blue light treatments could be quenched. The reduction in ROS levels mediated by 10 mM NAC in pHGFs treated with 60 J/cm^2 blue light was found to be significant, versus pHGFs receiving 60 J/cm^2 blue light treatment without NAC (Fig. 2A, $p < 0.001$).

Blue light produces minor effects on the transcription and synthesis of antioxidant genes

A broad panel of gene targets associated with antioxidant responses and regulation of cellular ROS levels, were further investigated in both pHGFs and pHGKs (Fig. 3). At 6 h post treatment, pHGFs showed a near 2-fold increase in NQO1 expression, in response to 3, 36 and 60 J/cm^2 blue light treatments (Fig. 3A). At 24 h post treatment, 2-fold increases in the expression of NQO1, GSR and GSS were observed in response to 36 and 60 J/cm^2 doses (Fig. 3B). Increases in KEAP1 were also noted in pHGFs in response to 36 J/cm^2 doses. However, these observed changes were deemed not statistically significant (all $p > 0.05$). No or limited changes in the expression of any of the targeted antioxidant genes were observed in pHGKs at 6 h post treatment (Fig. 3C-D). These included NFE2L2, which is the gene name for NRF2.

Noting small or minimal changes in mRNA levels in pHGFs, protein levels for NQO1, GSR and KEAP1 were additionally examined (Fig. 4). Immunoblots and subsequent quantification by densitometric analysis indicated minimal observable changes in the protein levels of these antioxidants upon blue light treatment for 6–24 h, which were determined to be not statistically significant (all $p > 0.05$).

Changes in antioxidant gene expression and metabolic activity are mediated by ROS

Having established the utility of 10 mM NAC as an effective ROS scavenger during 457 nm blue light treatments (Fig. 2), NAC was used to assess the contribution of ROS generated during blue light treatments upon the gene expression of the antioxidant GSR in pHGFs (Fig. 5). Treatment of pHGFs irradiated with 30 J/cm^2 and 60 J/cm^2 with 10 mM NAC, resulted in a 3-fold reduction in the mRNA expression of GSR, compared to pHGFs which received blue light treatments alone, although this was deemed to be not statistically significant ($p > 0.05$).

Discussion

The penetration depth of blue light through epidermal tissues is reportedly limited to approximately 1 mm [29]. As the light passes through the epithelial layers to the underlying laminar propria, light is scattered and absorbed by photo-receptors within the resident keratinocytes and fibroblasts [29, 30], with potential to influence cellular activity. Oral mucosal tissues are approximately 0.3 mm thick, thinner than dermal tissues, and while the bulk of the oral epithelium presents as a keratinising protective barrier, non-keratinised epithelial cells of the sulcular and junctional

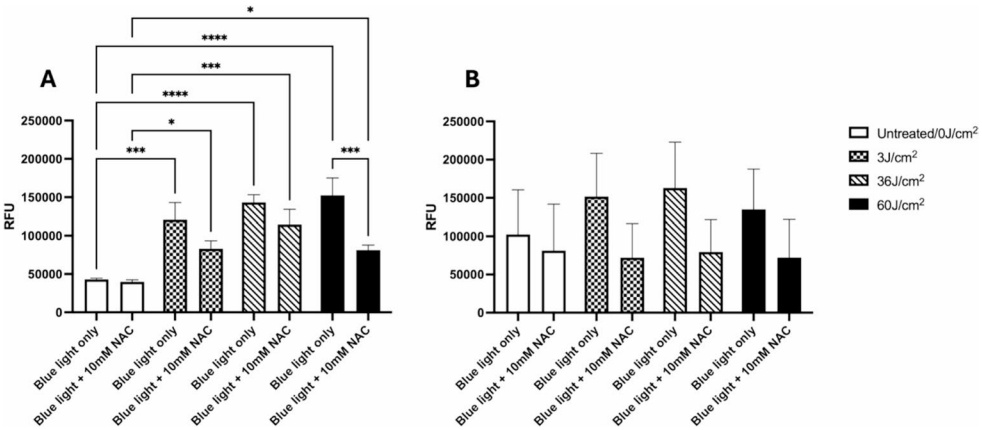

Fig. 2 Assessment of ROS generation following the blue light (457 nm) treatment of pHGFs (**A**) and pHGKs (**B**). ROS levels were determined immediately following blue light treatment and the oxidation of added H_2DCFDA resulting in the emittance of a green, fluorescent signal. ROS generation is reported as a value proportional to relative fluorescence units (RFU). N = 3, mean ± SD (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

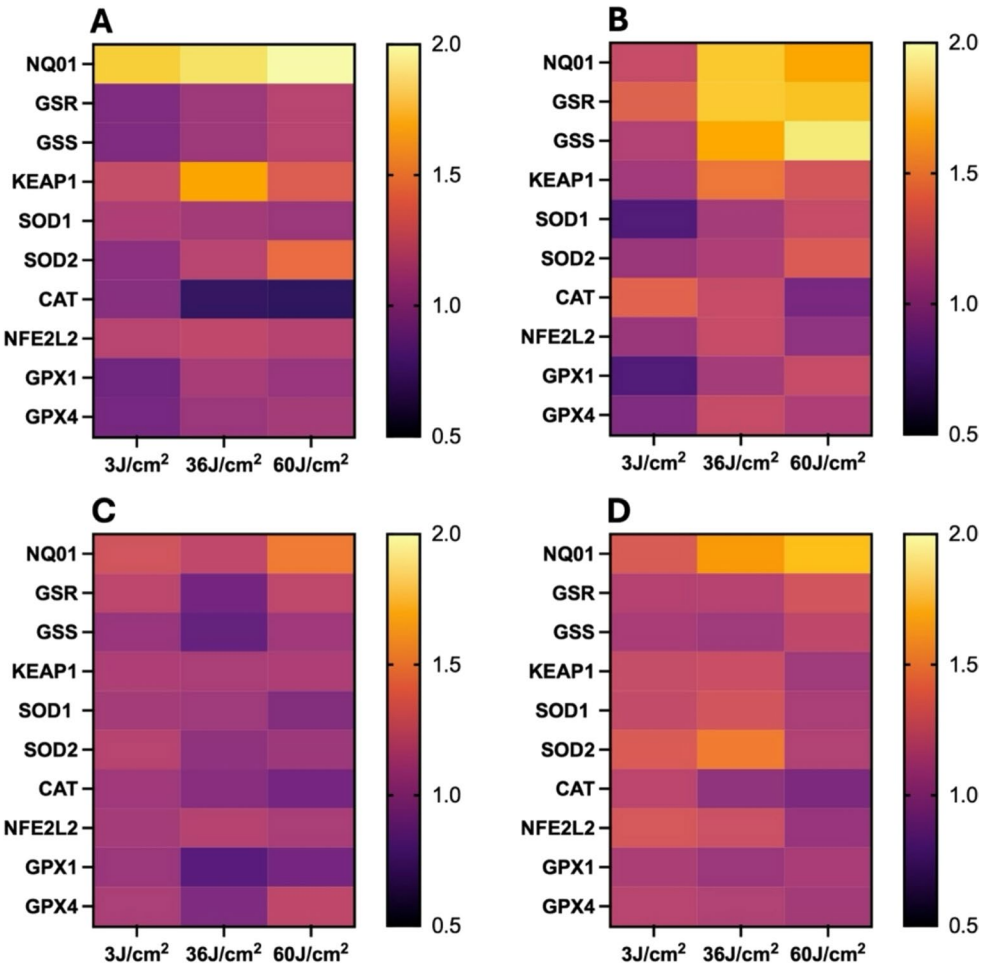

Fig. 3 Heatmaps depicting the fold changes in the gene expression of specific antioxidants (defined in Table 1), proposed to be upregulated through ROS generation and measured at 6 h (**A**, **C**) and 24 h (**B**, **D**) in pHGFs and pHGKs, respectively. Relative gene expression is displayed as colours ranging from black to yellow, according to the scale, where the highest fold change is shown in yellow. Expression of target genes in untreated samples is represented by 1 on the colour scale (N = 3)

epithelia surround the neck of the tooth [31, 32]. Consequently, any blue light treatment which has proven to be effective in reducing bacterial load in periodontal pockets [2–4], may also evoke cellular responses within surface facing keratinocytes, which may also penetrate through to the underlying fibroblasts populations that dominate these tissues. Within this study, we present novel data to indicate that blue light irradiation can increase the metabolic activity of pHGKs, with minimal effect for induction of apoptosis or ROS generation. Conversely, blue light irradiation is capable of significantly reducing the metabolic activity and increasing apoptosis of pHGFs, which our results would suggest is mediated by transient ROS generation.

The oral mucosa acts as a protective barrier and optimal wound healing is essential in the preservation of a functional healthy tissue. Keratinocytes play an important role in the constant re-epithelisation, supported by their high migratory and proliferative ability. Within the present study, irradiation of keratinocytes to blue light with fluence levels of 36 J/cm^2 and above increased the metabolic activity, with statistical significance. These fluences represented a range of irradiance levels (300 or 100 mW/cm^2) and duration (120–600 s), but responses were only dose-dependent upon the overall increasing level of fluence. Increases in metabolic activity detected by the reduction of Alamar Blue in the cytosolic environment directly correlate to an increase in the viable cell count, compared to untreated controls. Additionally, our results would indicate that following irradiation with blue light, pHGK do not experience significant oxidative stress, as near basal levels of ROS production were measured over the 30 min, following blue light treatment. Similarly, levels of LDH remained close to basal levels, suggesting blue light does not induce apoptosis. These observations align with previous studies that have determined the levels of enzymic antioxidants and products of oxidation within oral and dermal tissue biopsies [23]. Of note, immunodetection of SOD1, SOD2, SOD3 and catalase is considerably higher in the epidermal cells of both these tissues, compared with cells of the underlying laminar propria; a finding that correlates with the detection of significantly lower levels of oxidative stress-induced, biomolecular damage within epithelial tissues [23]. To the authors knowledge at time of publication, the results from the present study represent the first observation of the effect of blue light on the cellular behaviour on

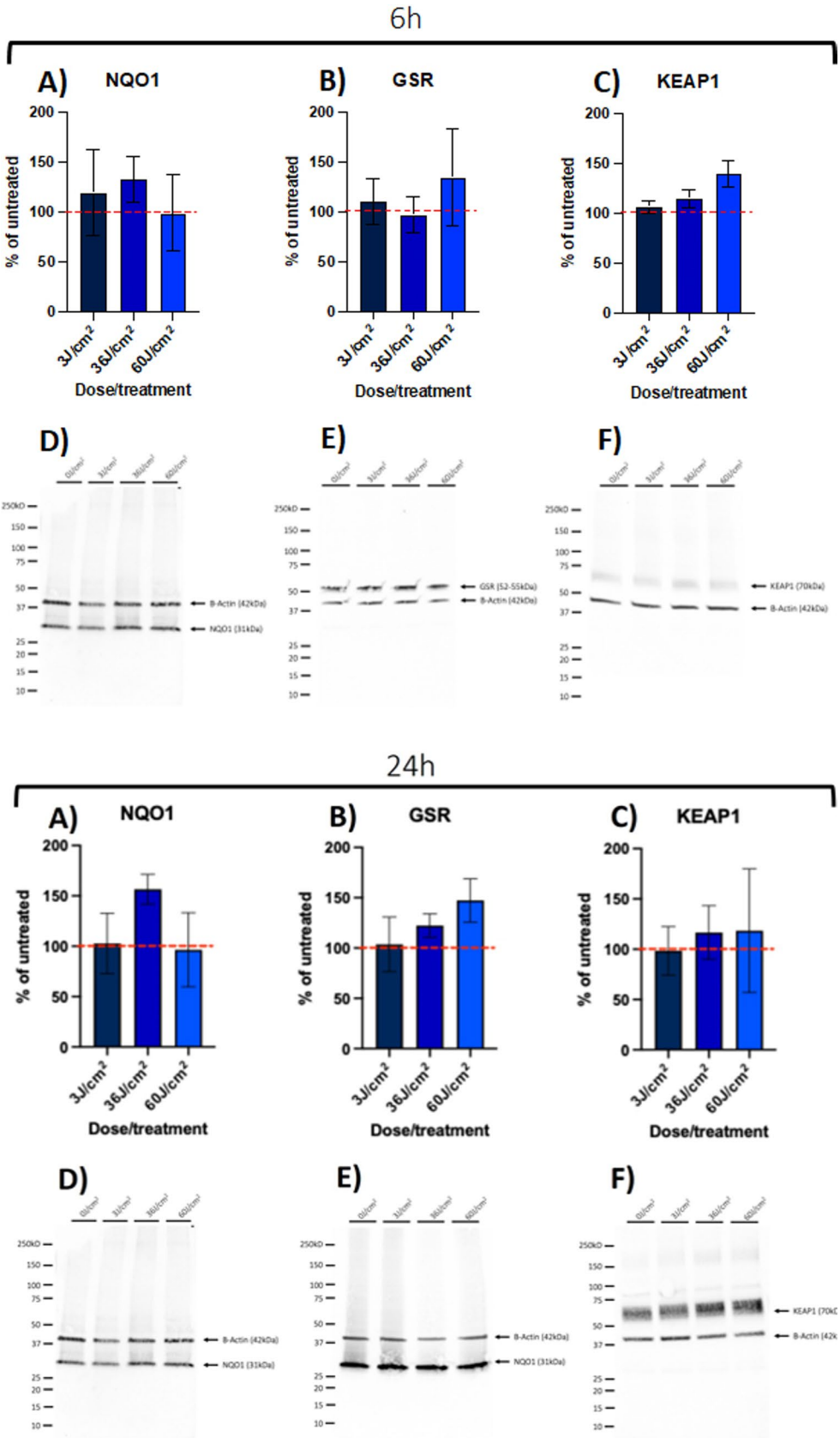

Fig. 4 Western blot and densitometry analysis of NQO1 (A, D) GSR (B, E) and KEAP1 (C, F) protein levels in pHGFs following blue light (457 nm) treatment. Analysis was performed on cell lysatesretrieved 6 h (top half of image) and 24 h (lower half of image) post-treatment. Western blot band intensity was normalised to the β-actin loading control, before calculating percentage changes in band intensity relative to untreated controls, represented by the dashed line. *N=3,* mean ± SD.All $p>0.05$.

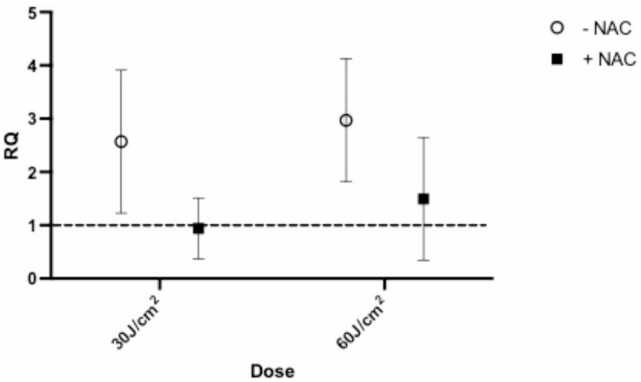

Fig. 5 Evaluation of the role which ROS play in regulating antioxidant gene expression in pHGFs post-blue light treatment. pHGFs were irradiated with 457 nm blue light in the presence and absence of 10 mM NAC. mRNA was isolated from pHGFs 6 h post-blue light treatment. GSR gene expression was normalised to the expression of GSR in non-irradiated cultures, which had received the same 10 mM NAC treatment. $N = 3$, mean ± SD

primary human gingival epithelial cells, but our results do align with similar studies which identified negligible effects of blue light on dermal keratinocytes, potentially reflecting genotypic and phenotypic differences between oral mucosal and dermal epithelial cells [18, 23, 33]. Collectively, these findings would suggest that epithelial cells of the oral mucosa possess good intracellular mechanisms to withstand blue light-derived, oxidative stress. However, this conclusion should be balanced against the observations that blue light can induce DNA damage in human dermal epithelial cells, which were reparable through base excision repair and nucleotide repair pathways [34]. Within dermal tissues, the generation of DNA damage has been linked with premature cell senescence and skin aging [34]. The nature of DNA damage caused by blue light in gingival epithelial cells is yet to be determined.

Within the oral mucosa, fibroblasts play important roles within wound healing; providing paracrine signalling between the different cell types, including the epithelial and immune cells and orchestrating angiogenesis, collagen synthesis and ECM formation. Moreover, fibroblasts of the oral mucosa are genotypically and phenotypically "younger" than dermal fibroblasts, which has been attributed to their preferential wound healing and minimal scarring properties influenced by differences in matrix metalloproteinase-mediated ECM remodelling and lower $TGF\beta_1$ secretion that limit myofibroblast differentiation, collagen deposition and fibrosis [24, 26, 35–37]. Noting different response to those observed by pHGKs, the irradiation of pHGFs resulted in a small dose dependent decrease in the metabolic activity. Although this decrease was only statistically different for cells irradiated with fluence of 30 J/cm^2, a corresponding increase in apoptosis is also witnessed, which may account for the decrease in viable cells. The observed differences in cellular responses of gingival epithelial and fibroblasts cells is also reported in dermal tissues, with fibroblasts more susceptible to blue light resulting in decreased proliferation and induced cytotoxicity [14, 18]. Opsins are intracellular light sensitive G protein-coupled receptors and following light activation opsins can activate a myriad of cell signalling pathways, including wound healing responses in skin [38, 39]. All four opsins have been reported to be present in the epidermal and dermal layers of the skin [38], with cells of the two layers likely to express different opsin profiles, resulting in light of variable wavelengths triggering different cell signalling pathways. However, it is notably that OPN3 (also known as encephalopsin/panopsin and sensitive to light within the 460–470 nm blue light range) are highly expressed in fibroblasts [38]. Such difference may partially account for the higher cytotoxic influences of blue light on fibroblasts cell populations. Differences in opsin profiles, may also partially account for the higher cytotoxic effects of 418 nm violet irradiation on both the pHGFs and pHGKs used in this study, with OPN1 activated by light with an approximate 425 nm wavelength [38, 39]. Violet light has been shown to cause higher levels of oxidative stress, lysosomal and mitochondrial damage which has the capacity to act as a stressor of cellular homeostasis through the accumulation of lipofuscin, a granular pigment product arising from oxidized lipids, proteins, and metal ions [40].

Cells irradiated in the presence of the ROS scavenger NAC, saw significant reductions in the generation of ROS, most evident in pHGFs at the higher dose of 60 J/cm^2. The presence of NAC during blue light treatment also reduced the generation of ROS in pHGKs, although observed differences between NAC treated and untreated cells did not reach statistical significance. NAC ameliorates oxidative challenges through the provision of sulfhydryl groups, which serve as a precursor to replenish GSH levels, thus driving GSH/GSSG ratios in favour of a reducing environment [41, 42]. The concentration of NAC during blue light treatment, therefore, dictates the amount of ROS scavenged. Nonetheless, our results suggest that blue light treatments do promote ROS production.

Blue light treatment can also disrupt intrinsic molecular function triggered by oxidative stress induced by ROS generation, most notably superoxide radical species ($O_2.^-$) generated via mitochondrial electron transport chain activity [43]. In alleviating the resultant oxidative stress, intracellular generation of ROS is also known to mediate a variety of cellular responses, such as NRF2 signalling that regulates antioxidant gene transcription [44]. In observing the detrimental effects of blue light in oral fibroblasts, this study continued to investigate changes in the gene transcription of a wide range of mitochondrial antioxidant candidates in pHGFs and pHGKs, identifying only minimal changes. Of

note, small increases (approximately 2-fold) were observed in mRNA levels for NQO1, GSR and GSS in pHGKs, which were slightly more pronounce in pHGFs. However, further analyses suggested that these increases did not yield a consequential change in the protein levels in the fibroblast populations examined within this study. Antioxidant levels for SOD1, SOD2 and catalase remained relatively unchanged. Immunohistochemical analysis for oral mucosal tissues have demonstrated the constitutive presence of these antioxidants associated with the epithelial cells and fibroblasts of the lamina propria [23], and these levels may have been appropriate to neutralise any transient production of ROS generated by blue light treatment. Similar justification may be true in considering no changes were noted for glutathione peroxidase 1 and 4.

All the above intracellular antioxidants are themselves regulated at a gene level by Nuclear Factor Erythroid 2-related Factor 2 (NRF2) [44, 45], and it is noted that blue light likewise did not induce any significant changes in the gene expression of this key regulatory transcription factor. However, the activity of NRF2 is tightly regulated by KEAP1. Whist no significant increases in KEAP1 were observed, under oxidative stress conditions, KEAP1 is known to be oxidised at reactive cysteine residues resulting in KEAP1 inactivation. This event subsequently results in the release of tethered cytosolic NRF2 which translocates to the nucleus, where it binds with antioxidant response elements to initiate gene transcription of genes, including NQO1 and GSS [45]. Within this study, observed by immuno-localisation of the protein NRF2 in the cytosol of pHGFs and pHGKs, we have obtained preliminary evidence to suggest that blue light treatment is associated with the movement of NRF2 to align with the cytoskeleton and engage with the nucleus (results not included), presumably in the activation of antioxidant response element genes.

Conclusions

Blue light treatments have become a useful tool in the treatment of a range of dermatological conditions, including such as actinic keratosis, acne, cutaneous infections, and psoriasis. Despite differences in the cell populations present in gingival tissues, this study would suggest that no major adverse effects were observed following blue light irradiation, nor a subsequent generation of excessive and potentially deleterious levels of ROS. This may be attributed to the valuable levels of antioxidants that are constitutively expressed in the epithelial layers of both dermal and oral tissues, which help maintain the functional integrity of these tissues [23]. Fluence levels of blue light examined in this study are relatively low, with cells exposed to tissue for no more than 1 min. However, fluence levels of 12 J/cm^2 have been demonstrated to be effective in reducing the mean survival of black-pigmented bacteria by 28.5% and 48.2% in plaque suspensions and biofilms, respectively [3]. Whilst this does not completely eliminate oral bacteria, it does demonstrate the potential for blue light treatments as a prophylaxis in hindering periodontal disease progression. However, it should be recognised that the photosensitive generation of ROS and subsequent antioxidant response of the cells in the oral mucosal remains complex, multifactorial, with many other cellular responses possible and significant further work is required to investigate other influences of blue light on other cell type and signalling pathways, including immune-inflammatory cells, vascular cells, and cytokines and growth factors, that are capable of influence gingival wound healing responses.

Pieper C, Lee EB, Swali R, Harp K, Wysong A. Effects of Blue Light on the Skin and Its Therapeutic Uses: Photodynamic Therapy and Beyond (2022) Dermatol Surg 48:802-808.https://doi.org/10.1097/DSS.0000000000003500.

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Author contributions EGC: Conceptualization, methodology, investigation, writing, review and editing; MS Conceptualization, methodology, review and editing; HR, investigation and review; MB Conceptualization, methodology, review and editing; RM Conceptualization, methodology, review, editing, supervisor; RJW Conceptualization, methodology, writing, review, editing and supervisor.

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Data availability Raw data generated as part of this study is available on request.

Declarations

Ethical approval Cells used in this study were from commercial sources, using the accompanying ethics approval.

Competing interests Two of the authors (Monique Stoffels and Matthias Born) are employees of Philips Oral Healthcare, the company that funded this study. However, they do not stand to gain financially from the outcomes of this research, and their involvement did not influence the objectivity or integrity of the study's findings.

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